GMR

The Original by FUNPEC-RP

DNA vaccine and DNA prime-protein boost with the *virB9* and *virB12* genes induced low level of protection against *Brucella abortus* infection in mice

M.A.B. Caitano-Bertolacci[1*], R.R.P. Bastos[2*], L.R. dos Santos[3], C.O. Soares[3], J.S.G. Rieger[1], C. Mantovani[1], M. de S. Santana[3]and G.M.S. Rosinha[3]

[1]Fundação Universidade Federal de Mato Grosso do Sul, Faculdade de Ciências Farmacêuticas, Alimentos e Nutrição, Campo Grande, MS, Brasil
[2]Fundação Universidade Federal de Mato Grosso do Sul, Faculdade de Medicina Veterinária e Zootecnia, Campo Grande, MS, Brasil
[3]Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa em Gado de Corte, Campo Grande, MS, Brasil

*These authors contributed equally to this study.

Corresponding author: G.M.S. Rosinha
E-mail: gracia.rosinha@embrapa.br

Genet. Mol. Res. 18 (2): gmr18295
Received March 07, 2019
Accepted May21, 2019
Published June 24, 2019
DOI http://dx.doi.org/10.4238/gmr18295

ABSTRACT. VIRB proteins from *Brucella* spp. constitute the type IV Secretion System (T4SS), a key virulence factor that mediates the intracellular survival of these bacteria. We investigated the immunogenicity and protection of proteins produced by the *virB9* and *virB12* genes in the DNA vaccine and DNA prime-protein boost strategies. Groups of 10 mice were vaccinated with pcDNA*virB9*, pcDNA*virB12*, pcDNA*virB9*+rVIRB9 or pcDNA*virB12*+rVIRB12. The latter two groups were vaccinated with the proteins rVIRB9 and rVIRB12, respectively, during the third immunization. Three weeks after the last immunization, six animals from each group were challenged intraperitoneally with *B. abortus* strain S2308, and the efficacy of the vaccines was calculated as the $\log_{10}$ of protection by subtracting the mean log CFU of the vaccinated group from the mean log CFU of the

negative control group (injected with sterile saline). Most of the vaccinated mice produced total IgG and the subclasses IgG1 and IgG2a against the respective protein, except for the mice vaccinated with pcDNA*virB12*. Cytokines IFN-γ and IL-10 were produced, but without a significant difference between the vaccinated and negative control groups. The vaccines did not induce significant levels of protection, in contrast to the immunization obtained with the S19 vaccine strain (Log_{10}, 1.48). In conclusion, the *virB9* and *virB12* genes of *B. abortus*, using DNA vaccine and DNA prime-protein boost strategies, were able to induce both humoral and cellular immune responses, but not enough to induce significant protection in the immunized mice. However, given the response in this system, further investigations using the *virB9* and *virB12* genes of *Brucella* spp., together with different immune modulators, are warranted. An effort should be made to direct and enhance the immune response, in order to identify a combination that stimulates a better immune response and, consequently, a better level of protection.

Key words: Bovine Brucellosis; *Brucella abortus;* DNA vaccine; *virB* operon; T4SS; *virB9*; *virB12*

INTRODUCTION

Brucellosis is one of the most well-known zoonosis worldwide caused by *Brucella spp*., a genus of Gram-negative bacteria. This disease causes high morbidity in animals and humans, leading to large economic losses and public health problems in many countries (Seleem et al., 2010).

Bovines are most commonly affected by the species *Brucella abortus*, causing the following reproductive problems; pre-term abortion, birth of weak calves, placenta retention, permanent infertility and perinatal mortality (Corbel, 2006; Neta et al., 2010). The disease also affects the reproductive system in bulls, causing orchitis, epididymitis, a reduction in the sperm quality and infertility (Neta et al., 2010). The Chronic form of brucellosis is related to the obligate intracellular lifestyle of the bacteria, which is capable of inhabiting different types of cells including macrophages and dendritic cells. (Comerci et al., 2001).

Vaccination continues to be the most effective way to control the disease (Olsen and Stoffregen, 2005). The current vaccines used to prevent bovine brucellosis are the live attenuated strains *B. abortus* RB51 and S19 (Goodwin and Pascual, 2016). However, these vaccines can cause abortion in immunized pregnant animals, interference in serological tests based on lipopolysaccharides and pathogenicity in humans (Dorneles et al., 2015; Yousefi et al., 2016; Cossaboom et al., 2018).

Immunization with DNA is considered a new approach to develop new vaccines against bovine brucellosis. It is considered a strategy capable of inducing a type 1 immune response (Th1) associated with IFN-γ producing $CD4^+$ T cells, $CD8^+$ cytotoxic T cells and IgG2a antibodies produced by B cells, all of which are necessary to eliminate infection by *B. abortus* (Golding et al., 2001). In addition, it is possible to enhance DNA vaccine immune responses by using a DNA *prime-protein boost* (Cassataro et al., 2007; Golshani et al., 2015). An initial immunization with DNA, followed by enhancement with recombinant

protein, can increase the efficacy of a vaccine against *Brucella* infection (Cassataro et al., 2007; Golshani et al., 2015).

One of the main virulence factors that mediates intracellular survival of different species of *Brucella* is the type IV secretion system (T4SS), encoded by the *virB* operon (*virB1* to *virB12*). Since its identification, T4SS from *Brucella* has been studied extensively. The results indicate that it plays a crucial role in the inhibition of the host innate immune response. However, at a stage following its intracellular survival, the immune response of the host is activated (Ke et al., 2015).

Bacteria use T4SS for genetic exchange and to translocate effector molecules to eukaryotic target cells. These effector molecules then target various host mechanisms and perpetuate the infection. Recent discoveries suggest the T4SS of *Brucella* spp. can provide effector molecules via AIM2 or NLRP3 to activate ASC inflammasomes leading to an activation of caspase-1 and innate resistance to *Brucella abortus* infection (Gomes et al., 2013; Ke et al., 2015).

Identifying the effectors secreted by *Brucella* through the T4SS and determining their target host pathways are critical for understanding their pathogenesis. A better understanding of these mechanisms will help to develop better vaccines against *Brucella* (Ke et al., 2015).

The expression of *virB* genes is intracellularly induced within the first hours after uptake of *Brucella* by macrophages (Sieira et al., 2004). Genes *virB9* and *virB12* encode proteins located on the surface of *Brucella* spp. Therefore, infected macrophages probably display peptides derived from VIRB proteins on the class II major histocompatibility complex (MHC-II) on the cell surface (Pollak et al., 2015). Despite not being necessary for bacterial intracellular survival, the VIRB12 protein has been described as highly antigenic, possibly contributing to immunogenicity (Rolán et al., 2008). Furthermore, VIRB9, which is also antigenic, and is necessary for the survival and replication of the pathogen within host cells (den Hartigh et al., 2008; Delpino et al., 2009; Pollak et al., 2015).

Due to the characteristics mentioned above and the highly conserved sequences of the *virB9* and *virB12* genes among *Brucella* species, two DNA vaccines were developed. The immunogenicity of the vaccines was evaluated, as well as their ability to induce protection on their own, or in combination with recombinant protein (DNA *prime-protein boost*).

MATERIAL AND METHODS

Mice

Female BALB/c mice aged 6 to 8 weeks were obtained from the Fundação Universidade Federal de Mato Grosso do Sul (Mato Grosso do Sul, Brazil). Animals were kept in a special facility at the Embrapa Gado de Corte (CNPGC) in controlled conditions (21 ± 2 ºC and 12-h light/dark cycles) and fed commercial feed and water *ad libitum*. They were randomly grouped and identified. The experimental procedures were previously approved by the Ethics Committee for the use of Animals at the Embrapa Gado de Corte (protocol nº 016/2014).

Construction and preparation of plasmid DNA vaccines

DNA vaccines were constructed by cloning *virB9* and *virB12* genes into the pcDNA^{TM}3.1(+) (*Invitrogen*®) vector. The amplification of *virB9 and virB12*genes from *B. abortus* was carried out by polymerase chain reaction (PCR), using oligonucleotides containing the restriction sites for *Bam*HI and *Hind*III (Table 1). The oligonucleotides were designed using DNASTAR®, based on the genomic sequence of the *B. abortus* strain (AF 226278.1). PCR products were inserted into the pcDNA^{TM}3.1(+) (*Invitrogen*®) vector and positive clones were confirmed by restriction analysis and DNA sequencing. Recombinant plasmids pcDNA*virB9* and pcDNA*virB12* were produced on a large scale and purified using an EndoFree Giga Kit (*Qiagen* Inc., Valencia, CA, USA) according to the manufacturer's protocols.

Table 1. Sequences of oligonucleotides used to amplify genes*virB9*and *virB12*from *Brucella abortus* by PCR.

Oligonucleotide	Sequence	Restriction site
pcDNA*virB9*F	5' CAAAAGCTTACGATGGAAAGATTCCTGCTTGCGTGC 3'	*Hind*III
pcDNA*virB9*R	5' CAAGGATCCTCATTGCAGGTTCTCCCCG 3'	*Bam*HI
pcDNA*virB12*F	5' CAAAAGCTTACGATGGGCACATTGGTTATGGTCG 3'	*Hind*III
pcDNA*virB12*R	5' CAAGGATCCTTACTTGCGTAAAATTTCGAT 3'	*Bam*HI

Expression and production of recombinant proteins VIRB9 and VIRB12

Genes *virB9* and *virB12* from *B. abortus*, ligated into pET-47b plasmid (Novagen®), were introduced into competent *E. coli* TOP10 F' for storage and culture. Stored strains were cultured in liquid LB broth and recombinant plasmids were extracted and purified using a *Wizard miniprep*™ kit (Promega®). *Escherichia coli* Rosetta was transformed using recombinant plasmids and the gene expression was induced by an incubation of liquid LB broth supplemented with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, *dioxane free, Ludwig Biotec*) at 37°C for 4 h. rVIRB9 and rVIRB12 proteins were purified by nickel affinity chromatography using a AKTA Purifier 10 chromatographer equipped with a *His Trap Sepharose HP column (GE Healthcare*) and 1x phosphate-buffered saline as the eluent, at pH 7.2 (PBS) for 72 h at 4ºC. Purification and sample quality were performed by 12% SDS-PAGE electrophoresis and Western blotting, by using Monoclonal Anti-polyHistidine, Clone HIS-1 (Sigma-Aldrich). The antibody was conjugated to peroxidase (Anti-Mouse IgG (Fc specific) in goat) at a 1:3000 dilution. The concentration of protein was measured by the Bradford method (Bradford, 1976).

Mice Immunization

Controls were injected with phosphate buffered saline (PBS). Mice were separated into control (PBS and S19 vaccine) and experimental (pcDNA*virB9*, pcDNA*virB12*, pcDNA*virB9*+rVIRB9 and pcDNA*virB12*+rVIRB12) groups, both containing 10 animals. The animals were vaccinated in 21-day intervals (day 0, 21 and 42). In the groups administered with DNA vaccines, each animal was intramuscularly

inoculated with 100 µg of each plasmid in 100 µL of PBS (50 µL was injected into each tibialis anterior muscle). For the DNA *prime-protein boost* strategy, mice received two 100 µg doses of the DNA vaccine on days 0 and 21, followed by a third 50 µg dose of the respective recombinant protein prepared with Montanide adjuvant (*Montanide™ ISA 61 VG*, *Seppic*) by subcutaneous injection in the dorsal region on day 42. An intraperitoneal inoculation of $5x10^4$ colony forming units (CFU) of *B. abortus* S19 vaccine was administered to the animals in the positive control group on day 42 of the experiment. For the negative controls, 100 µL of PBS was administered to the animals on all three days of immunization.

Protection Analysis

Three weeks after the last immunization, six mice of each group were challenged with $2x10^5$ CFU of *B. abortus* strain S2308 by intraperitoneal injection. Mice were sacrificed by cervical dislocation at 3 weeks post-infection and their spleens were removed aseptically. Each spleen was homogenized in PBS, serially diluted and cultured in Petri dishes containing tryptone soy agar (TSA, *Oxoid*) for 72 h at 37°C. The number of *Brucella* colonies was expressed as the mean $\log_{10}$ CFU in each spleen. Log_{10} units of protection were obtained by subtracting the mean $\log_{10}$ CFU of the experimental group from the mean $\log_{10}$ CFU of the corresponding negative-control group.

Immunoglobulin Production

Retro-orbital bleeding was performed in mice on days 0, 21, 42 and 63 and serum was collected and stored at -20°C until further use. The production of total specific IgG, as well as the isotypes IgG1 and IgG2a, were detected by the enzyme-linked immunosorbent assay (ELISA). Purified rVIRB9 and rVIRB12 in 2% sodium dodecyl sulfate (SDS) were used as antigens and adsorbed on polystyrene 96 well plates (*Costar*® 3590) in a 1:400 dilution in 0.05 M carbonate/bicarbonate buffer at a pH of 9.6, followed by incubation in a humid chamber at 4ºC for 16 h. Plates were washed with 0.05% PBS-Tween 20 (PBS-T) and blocked with 5% powdered skimmed milk in PBS-T for 1 h at 37°C.

After consecutive washes with PBS-T, sera (1:50) were added in duplicate to each plate. The plates were incubated for 1 h at 37°C, then washed with PBS-T. Anti-Mouse IgG-Peroxidase conjugate (*Sigma-Aldrich*®) was added to the plates in a 1:10.000 dilution, followed by another incubation for 1 h at 37°C. To evaluate IgG1 and IgG2a isotypes, rVIRB9 and rVIRB12 plates were blocked for 2 h at room temperature with 10% fetal bovine serum (FBS, Cultilab) in 1x PBS. Following PBS-T washes, different concentrations of sera (1:100, 1:300, 1:900, 1:2700, 1:8100, 1:24300, 1:72900, 1:218700) were added to determine the final titers (Frey et al., 1998). The plates were incubated for 1 h at room temperature and washed with PBS-T. Anti-IgG1 and Anti-IgG2a (*BD Biosciences*®) antibodies diluted to 1:1000 in PBS1x/FBS were then added to the plates and incubated for 2 h. The plates were then washed with PBS-T and

incubated with Avidin (*Sigma-Aldrich*®) (1:2000 in PBS1x/FBS) for 45 min at room temperature. Enzymatic reactions were carried out by adding the chromogen o-phenylene diamine (OPD) (*Sigma-Aldrich*®), following the manufacturer's instructions. The plates where then incubated in the dark and the colorimetric reaction was stopped by adding 2.5 N sulfuric acid (H_2SO_4). The optical density (DO) was obtained by measuring the absorbance at a wavelength of 490 nm using a spectrophotometer (Bio-Tek Instruments).

Cytokine detection

The levels of IFN-γ and IL-10 released into the supernatants of splenocyte culture were quantified by capture-ELISA. Thirty days after the final immunization, four mice from each group were euthanized and splenectomized aseptically. Duplicate spleen cell suspensions were prepared for each animal in RPMI culture medium (*Sigma*®) supplemented with 10% FBS and gentamicin (10 μg/mL). In 24 well plates, 2 x 10^6 cells per well of splenocytes were harvested and stimulated with 5 μg/mL of recombinant rVIRB9 or rVIRB12 protein. Concanavalin A (5 μg/mL) was used as a positive control for lymphocyte stimulation. A supplemented RPMI medium free of stimulating agents was used as a negative control. The rVIRB9, rVIRB12, Con A and RPMI medium stimuli were all previously treated with polymyxin B (10 μg/mL) for 30 min under constant agitation, followed by incubation for 48 h at 37ºC in a humid environment (5% CO_2). After incubation, the culture supernatants were collected and stored at -20°C. Cytokine quantification was carried out using a commercial capture ELISA system (*BD Biosciences*®), following the manufacturer's instructions, with the exception of using the SIGMAFAST™ OPD (*Sigma-Aldrich*®) chromogen. Optical density was measured by absorbance at 490 nm using a spectrophotometer.

Statistical analysis

Statistical analyses of normality and ANOVA followed by a Tukey's or Dunn's post-tests were carried out using the *GraphPad Prism* version 6. Differences were considered significant at a 95% confidence level ($P < 0.05$).

RESULTS

Development of DNA vaccines and production of recombinant proteins

The pcDNA*virB9* and pcDNA*virB12* constructs were verified by restriction analysis followed by automatic DNA sequencing. rVIRB9 and rVIRB12 proteins were obtained after 4 h of cultivation with IPTG (Figures 1A and B). After purification, rVIRB9 and rVIRB12 had approximate molecular weights of 32 and 19 kilodaltons (kDa), respectively (Figure 1C). The specificity of the vaccine was confirmed by anti-histidine monoclonal antibody (data not shown).

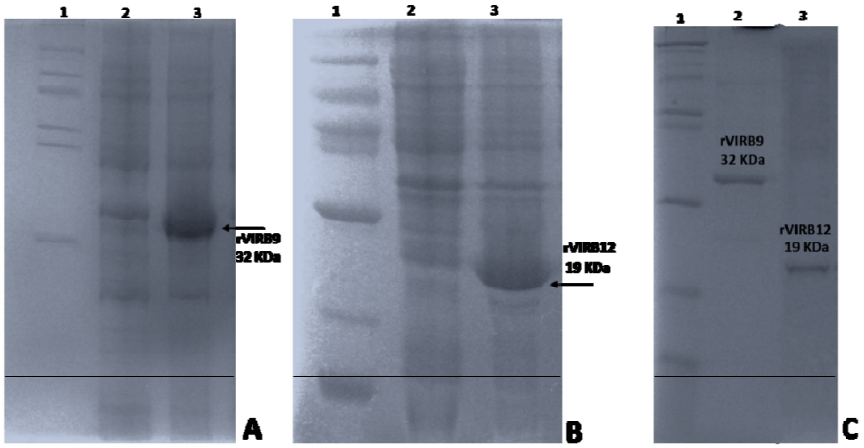

Figure1. Production and purification of rVIRB9 and rVIRB12. Electrophoresis in 12% SDS-PAGE stained with Coomassie Blue R-250. (A) Induction of rVIRB9 gene expression: Lane 1 - protein marker -Invitrogen (p-6649). Lane 2 - *Escherichia coli* protein profile prior to induction with IPTG. Lane 3 -Production of rVIRB9, 4 h after addition of 1mM of IPTG. (B) Induction of rVIRB12 gene expression: Lane 1 - protein marker - GE (17-0446-01). Lane 2 - *Escherichia coli* protein profile prior to induction with IPTG. Lane 3 - Production of rVIRB12, 4 h after addition of 1mM of IPTG. (C) rVIRB9 and rVIRB12 after His-Trap purification (GE Healthcare). Lane 1- protein molecular weight marker - Invitrogen (p-6649). Lane 2 - purified rVIRB9 protein (~32 kDa). Lane 3 - purified rVIRB12 protein.

Immunoglobulin Production

The production levels of IgG and of the isotypes IgG1 and IgG2a were evaluated in serum samples collected on day 0 (before immunization), 21, 42 and 63 (post-immunization). In mice immunized with pcDNA*virB9* and pcDNA*virB9*+rVIRB9 the total IgG production was observed on days 21, 42 and 63 (Figure 2A and B). Mice from the pcDNA*virB12*+rVIRB12 group produced IgG, but only after the third immunization with the recombinant protein. The pcDNA*virB12* group did not produce specific IgG against rVIRB12 (Figure 2C).

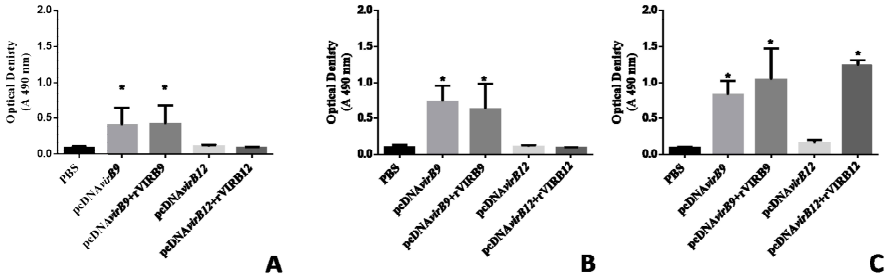

Figure 2. Production of specific anti-rVIRB9 and anti-rVIRB12 IgG in immunized mice. Theserum of each mouse was diluted 1:400 and used in ELISA. (A) Blood of immunized mice was collected by retro orbital bleeding 21 days after the first immunization. (B) Blood of immunized mice was collected by retro orbital bleeding 42 days after the first immunization and (C) Blood of immunized mice was collected by retro orbital bleeding 63 days after the first immunization. Asterisks (*) indicate significant differences ($P < 0.05$) compared to the control group (PBS).

Th1 and Th2 responses were specified by IgG2a and IgG1 isotypes, respectively. IgG1 and IgG2a levels specific for rVIRB9 or rVIRB12 were determined in sera obtained on the last collection (63 days following the 3rd dose). Mice from pcDNA*virB9* and pcDNA*virB9*+rVIRB9 groups presented the following antibody isotypes; IgG1 and IgG2a, there was however, no significant difference between the isotypes (Figure 3A). On the other hand, animals in the pcDNA*virB12*+rVIRB12 group produced significant IgG1 ($P < 0.05$), despite not producing an IgG2a antibody. Neither subgroup of antibodies was produced by animals in the pcDNA*virB12* group (Figure 3B).

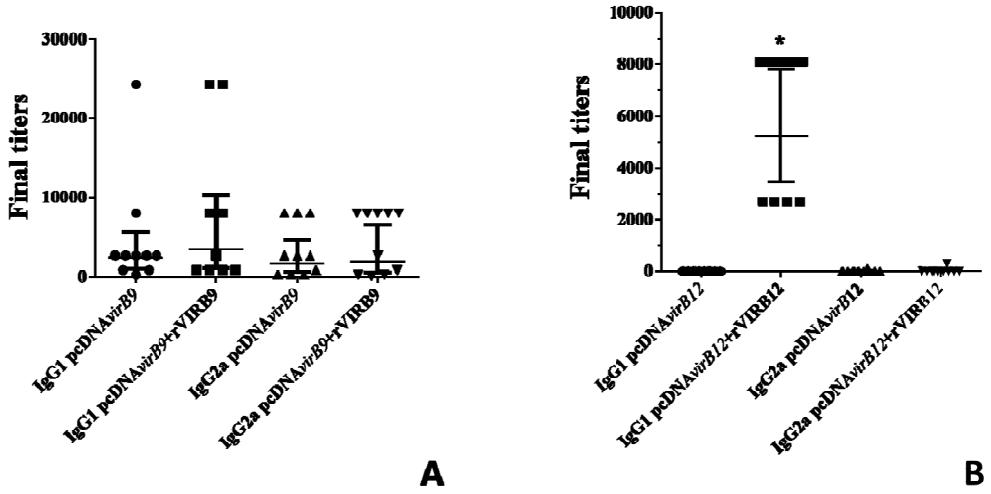

Figure 3. Final titers of IgG1 and IgG2a isotypes in mouse sera. (A) IgG1 and IgG2a antibodies specific for rVIRB9 in mice injected with pcDNA*virB9* vaccine or pcDNA*virB9*+rVIRB9 booster vaccine. (B) IgG1 and IgG2a antibodies specific for rVIRB12 in mice injected with pcDNA*virB12*vaccine or pcDNA*virB12*+rVIRB12 booster vaccine. Values represent the reciprocal dilution of final titers (*) indicates significant difference ($P < 0.05$) in geometric means with 95% CI (Dunn's test) between titers of IgG1 and IgG2a.

Cellular Immune Response

The profile of IFN-γ and IL-10 cytokines secreted in the culture of spleen cells from all experimental groups was evaluated using capture-ELISA. No differences were observed in the production of IFN-γ and IL-10 cytokines between the test and control (PBS) groups (Figure 4A and B).

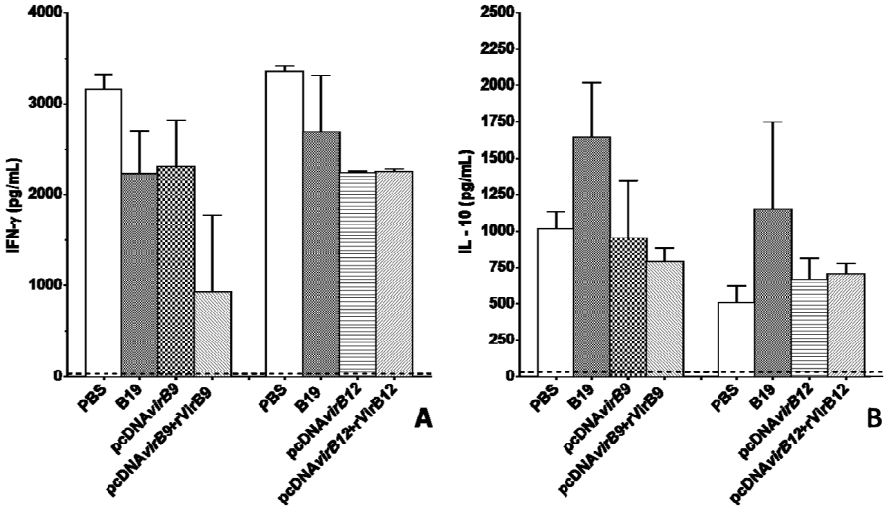

Figure 4. Determination of concentration of cytokines IFN-γ and IL-10. The supernatant of the spleen cell culture from mice immunized with experimental vaccines and control was used to determine concentrations of IFN-γ and IL-10. (A) Levels of IFN-γ produced by mice immunized with pcDNA*virB9*, pcDNA*virB*9+rVIRB9, pcDNA*virB12* and pcDNA*virB12*+rVIRB12. (B) Levels of IL-10 produced by mice immunized with pcDNA*virB9*, pcDNA*virB9*+rVIRB9, pcDNA*virB12* and pcDNA*virB12*+rVIRB12. Dashed line represents cutoff.

Level of Protection

In this experiment, the protective capacity of the vaccines was determined by exposing the immunized mice to 2 x 10^5 CFU of *B. abortus* S2308. The vaccine efficacy was calculated as the $\log_{10}$ of protection. Results showed that pcDNA*virB9*, pcDNA*virB12*+rVIRB12 and pcDNA*virB12* offered lower protection against *B. abortus* S2308, with 0.57, 0.30 and 0.21 $\log_{10}$ units of protection ($P > 0.05$), respectively. The immunization with pcDNA*virB9*+rVIRB9 did not induce protection (Table 2). The vaccinal S19 strain produced a significant increase of 1.48 $\log_{10}$ units of protection compared to the control animals ($P < 0.05$). The results showed no significant difference in CFU between the test groups and the PBS control group.

Table 2. Protection of mice challenged with 2 x 10^5 CFUs of *Brucella abortus* S2308 after immunization with DNA vaccines and booster with respective recombinant proteins.

Vaccine	$\log_{10}$ of CFUs of *B. abortus* S2308 in spleen (Mean ± SD)[a]	Protection units[b]
PBS	6.68 ± 0.11	-
S19	5.20 ± 0.57	1.48*
pcDNA*virB9*	6.11 ± 0.33	0.57
pcDNA*virB9*+rVIRB9	6.63 ± 0.19	0.05
pcDNA*virB12*	6.47 ± 0.41	0.21
pcDNA*virB12*+rVIRB12	6.37 ± 0.27	0.30

[a]Number of bacteria in spleen (CFUs/spleen) represented as mean of $\log_{10}$ of CFUs ± SD per group. [b]Protection units obtained by subtracting mean CFU log/spleen of groups immunized with experimental vaccines by mean log CFU of control group (PBS).*$P < 0.05$, significant difference compared to control group (PBS) and other groups.

DISCUSSION

New vaccines against brucellosis are essential in order to overcome the significant disadvantages of the live vaccines currently in use (Sislema-Egas et al., 2012). Since *Brucella* is an obligate intracellular pathogen residing in macrophages and dendritic cells, the vaccination regimen and the immunogens chosen should take into account the humoral and cellular (Th1) immune responses in order to achieve protection (Baldwin and Goenka, 2006).

Type IV secretion systems (T4SSs) are membrane-associated transporter complexes used by various bacteria and are involved in horizontal DNA transfer to other bacteria and eukaryotic cells. Furthermore, they are also involved in the uptake and release of DNA to the extracellular milieu, in the secretion of toxins, and in the injection of virulence factors into eukaryotic host target cells by various mammalian pathogens (Backert and Meyer, 2006).

Most of the secreted and/or membrane-bound bacterial proteins are considered to be antigenic and immunogenic. This could be the case for VIRB9 and VIRB12, which are both located on the cell surface and are part of the T4SS - *virB* operon of the *Brucella*, which is critical for persistence and intracellular survival (Rolán and Tsolis, 2008). They also provide effector molecules via AIM2 or NLRP3 to activate ASC inflammasomes and lead to the activation of caspase-1 and innate resistance to *Brucella abortus* infection (Gomes et al., 2013).

Studies have shown that VIRB12 is produced during the infection. As a result of this it is considered a serological marker (Mirkalantari et al., 2017; Rolán et al., 2008) in both experimental and natural infections (Mirkalantari et al., 2017).

Pollak et al. (2015) reported that splenocytes from mice immunized with VirB7 or VirB9 proteins from *Brucella* responded to their respective antigens with significant and specific production of IFN- γ, whereas interleukin-4 (IL-4) was not detected. After being experimentally exposed to the virulent *Brucella abortus* strain the spleen bacterial load was almost 1 log lower in mice immunized with VirB proteins than in unvaccinated animals. Additionally, they observed that dogs immunized with VirB7 or VirB9 produced IFN-γ after antigenic stimulation, suggesting the elicitation of a Th1 specific response to VirB. In this context, an analysis of the immunogenicity and ability of the proteins encoded by *virB9* and *virB12* genes to induce protection was conducted to develop a DNA vaccine and recombinant protein booster strategies.

In our study, the DNA vaccine enhanced by the recombinant protein booster induced a specific humoral immune response against rVIRB9 and rVIRB12, but it offered low protection for the mice against infection. Specific antibodies are important in reducing the initial phase of a *Brucella* infection, but a strong humoral immunity unaccompanied by cell-mediated immunity (CMI) cannot provide total protection against the *Brucella* organism (Lalsiamthara and Lee, 2017). In some situations, inoculation with “naked” plasmid DNA has been shown to not produce an immune response as strong as either purified protein or even a commercial *Brucella* vaccine (Cassataro et al., 2007; Golshani et al., 2015). DNA vaccines may be less potent than live attenuated vaccines because DNA is not as evenly distributed among initially transfected cells, while the number of cells infected with the live attenuated microorganism increases as it replicates (Kano et al., 2007). The route of administration should also be taken into account. Although DNA vaccines have

been highly successful in inducing immune responses in all tested routes of administration, some lead to higher antigen expression than others (Kano et al., 2007).

All strategies evaluated in our study presented an IgG2a/IgG1 ratio below 1, indicating a Th2 dominant immune response. Humoral immunity is essential for a preventive response of the plasmid DNA vaccines in fighting infections and IgG2a is the predominantly induced isotype (Oñate et al., 2003). On the other hand, the production of specific IgG1 immunoglobulins depends partly, on the presence of IL-4 produced by the Th2 lymphocyte subpopulation (Snapper et al., 1988). Despite this cytokine not being evaluated in this study, it possibly played a role in the induced immune response, due to the presence of IgG1. For intracellular pathogens, such as *B. abortus*, a Th2 response is less effective at combatting the infection.

Protection against *B. abortus* infection requires the induction of a Th1- type immune response, in which IFN-γ is a main cytokine for host control of brucellosis (Brandão et al., 2012). IL-10 is an important component in acquiring cellular resistance and contributes specifically to the control of *Brucella* multiplication (Gorvel and Moreno, 2002). IFN-γ produced by Th1 lymphocytes is important for modulating B cells to secrete IgG2a specific antigen (Nagata et al., 2004). Although in this study differences were not found in the production of these cytokines, following immunization and stimulation *in vitro* cell. It is therefore reasonable to assume that the induction of IgG2a antibodies indicates the presence of IFN-γ in groups immunized with the *virB9* gene in both strategies tested, as also observed by Pollak (Pollak et al., 2015).

Gomes et al. (2013) determined that the bacterial type IV secretion system *virB* is required for full inflammasome activation in macrophages during infection. It was also demonstrated that AIM2 acts as an important receptor for *Brucella* DNA in dendritic cells, which is essential in caspase-1-mediated cytokine processing, and controlling infections (Franco et al., 2019). As a result, it is possible to suggest that the *virB9* and *virB12* genes and their respective proteins in this study, were not recognized by the AIM2 cytoplasmic receptor. Therefore, there is no activation of the inflammasome in macrophages, necessary for the production of IFN-γ.

VirB genes have been studied and also indicated for use in vaccines against *Anaplasma* spp. Araújo et al. (2008) reported that bovine sera infected with *A. marginale* recognize the recombinant VIRB9 and VIRB10 proteins. In addition, IgG2 immunoglobulin from naturally infected cattle also react with these proteins, indicating their use in recombinant vaccines against this rickettsia. The recombinant VIRB9-1 and VIRB9-2 proteins of *A. marginale* used with nano-formulations in mice immunization showed high levels of antibody titers and strong T cell responses. Furthermore, the mixed nano-formulation of the two proteins also stimulated high-level memory responses in bovine T cell proliferation assays (Zhao et al., 2016).

In a recent study, the *virB9* and *virB10* genes of *Anaplasma phagocytophilum* were used in the DNA prime-protein boost strategy. The results showed that mice vaccinated with *virB10*,a subdominant conserved antigen that is a component of the T4SS, not only induced antigen-specific humoral and T cell mediated responses, but also elicited partial protection against challenge with *A. phagocytophilum* (Crosby et al., 2018).

The use of the live S19 vaccine in the immunization of mice resulted in the highest protection index observed after the challenge with the *B. abortus* strain S2308. The ability of live vaccines, such as S19, in controlling infections is most likely a direct effect of the

capacity of the bacteria to infect the host cell, and to allow the immune system access to a variety of antigens that jointly contribute to a stronger and more extensive response. However, this vaccine induces abortions in pregnant animals and is virulent for humans; moreover, it elicits anti-*Brucella* antibodies that interfere with serodiagnosis (Lalsiamthara and Lee, 2017).

Subunit vaccines, including DNA vaccines and recombinant proteins, present similar characteristics and advantages over the live S19 vaccine such as: being highly safe; no residual virulence; and provide the strategy known as differentiated infected from vaccinated animals (DIVA) (Lalsiamthara and Lee, 2017). There are however, some disadvantages which include poor immunogenicity when used alone, and also the requirement of adjuvants to elicit a protective and long-lasting immune response.

This study is the first report on the use of the *virB9* and *virB12* genes as DNA vaccines against *Brucella abortus*. Our results indicate that the vaccine strategies tested with these genes were able to induce both humoral and cellular immune responses, but not enough to induce a significant protection for the immunized mice. Therefore, further studies will be carried out using these molecules, including the use of nanoparticles as adjuvants, as well as different inoculation routes and the use of recombinant protein as a vaccine.

ACKNOWLEDGMENTS

This study was funded by the Embrapa Beef Cattle (grant number 03.15.00.069.00.00); Foundation for Support of the Development of Teaching, Science and Technology of the State of Mato Grosso do Sul - FUNDECT (grant number 23/200.237/2014) and the Brazilian Coordination for the Advancement of Higher Education Personnel (CAPES).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Araújo FR, Costa CM, Ramos CAN, Farias TA, et al. (2008). IgG and IgG2 antibodies from cattle naturally infected with *Anaplasma marginale* recognize the recombinant vaccine candidate antigens VirB9, VirB10, and elongation factor-Tu. *Mem. Inst. Oswaldo Cruz.* 103: 186-190.

Avila-Calderón ED, Lopez-Merino A, Sriranganathan N, Boyle SM, et al. (2013). A history of the development of *Brucella* vaccines. *Biomed Res. Int.* 2013: 743509.

Backert S and Meyer TF (2006). Type IV secretion systems and their effectors in bacterial pathogenesis. *Curr. Opin. Microbiol.* 9: 207-217.

Baldwin CL and Goenka R (2006). Host immune responses to the intracellular bacteria *Brucella*: does the bacteria instruct the host to facilitate chronic infection? *Crit. Rev. Immunol.* 26: 407–42.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Brandao AP, Oliveira FS, Carvalho NB, Vieira LQ, et al. (2012). Host susceptibility to *Brucella abortus* infection is more pronounced in IFN gamma knockout than IL-12/beta2-microglobulin double-deficient mice. *Clin. Dev. Immunol.* 2012: 589494.

Cassataro J, Velikovsky CA, Bruno L, Estein SM, et al. (2007). Improved immunogenicity of a vaccination regimen combining a DNA vaccine encoding *Brucella melitensis* outer membrane protein 31 (Omp31) and recombinant Omp31 boosting. *Clin. Vaccine Immunol.* 14: 869–874.

Comerci DJ, Martinez-Lorenzo MJ, Sieira R, Gorvel JP, et al. (2001). Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. *Cell. Microbiol.* 3: 159–168.

Copin R, Vitry MA, Hanot Mambres D, Machelart A, et al. (2012). In Situ Microscopy Analysis Reveals Local Innate Immune Response Developed around *Brucella* Infected Cells in Resistant and Susceptible Mice. *PLoS Pathog.* 8: e1002575.

Corbel MJ (2006). Brucellosis in humans and animals Food and Agriculture Organization of the United Nations. Geneva: World Health Organization.

Cossaboom CM, Kharod GA, Salzer JS, Tiller RV, et al. (2018). Notes from the Field: *Brucella abortus* Vaccine Strain RB51 Infection and Exposures Associated with Raw Milk Consumption - Wise County, Texas, 2017. *Morb. Mortal. Wkly. Rep*. 67: 286.

Crosby F, Lundgren AM, Hoffman C, Pascual DW, et al. (2018). VirB10 vaccination for protection against *Anaplasma phagocytophilum*. *BMC Microbiology.* 18: 217.

Delpino MV, Comerci DJ, Wagner MA, Eschenbrenner M, et al. (2009). Differential composition of culture supernatants from wild-type *Brucella abortus* and its isogenic VirB mutants. *Arch. Microbiol.* 191: 571–581.

den Hartigh AB, Rolan HG, de Jong MF and Tsolis RM (2008). VirB3 to VirB6 and VirB8 to VirB11, but Not VirB7, Are Essential for Mediating Persistence of *Brucella* in the Reticuloendothelial System. *J. Bacteriol.* 190: 4427–4436.

Dorneles EMS, Teixeira-Carvalho A, Araújo MSS, Sriranganathan N, et al. (2015). Immune response triggered by *Brucella abortus* following infection or vaccination. *Vaccine.* 33: 3659–3666.

Franco MMSC, Marim FM, Alves-Silva J, Cerqueira D, et al. (2019). AIM2 senses *Brucella abortus* DNA in dendritic cells to induce IL-1b secretion, pyroptosis and resistance to bacterial infection in mice. *Microbes Infect.* 21: 85-93.

Frey A, Di Canzio J and Zurakowski D (1998). A statistically defined endpoint titer determination method for immunoassays. *J. Immunol. Methods.* 221: 35–41.

Golding B, Scott DE, Scharf O, Huang LY, et al. (2001). Immunity and protection against *Brucella abortus*. *Microbes Infect.* 3: 43–48.

Golshani M, Rafati S, Siadat SD, Nejati-Moheimani M, et al. (2015). Improved immunogenicity and protective efficacy of a divalent DNA vaccine encoding *Brucella* L7/L12-truncated Omp31 fusion protein by a DNA priming and protein boosting regimen. *Mol. Immunol.* 66: 384–391.

Gomes MTR, Campos PC, Oliveira FS, Corsetti PP, et al. (2013). Critical Role of ASC Inflammasomes and Bacterial Type IV Secretion System in Caspase-1 Activation and Host Innate Resistance to *Brucellaabortus* Infection. *J Immunol.* 190: 3629-3638.

Goodwin ZI and Pascual DW (2016). Brucellosis vaccines for livestock. *Vet. Immunol. Immunopathol.* 181: 51–58.

Gorvel JP and Moreno E (2002). *Brucella* intracellular life: from invasion to intracellular replication. *Vet. Microbiol.* 90: 281–297.

Kano FS, Vidotto O and Vidotto MC (2007). Vacina de DNA: aspectos gerais e sua aplicação na medicina humana e veterinária. *Semina: Ciências Agrárias.* 28: 709-726.

Ke, YH, Wang Y, Li W and Chen Z (2015). Type IV secretion system of *Brucella* spp. and its effectors. *Front Cell. Infect. Microbiol.* 5: 72. DOI: 10.3389/famb.2015.00072.

Lalsiamthara J and Lee JH (2017). Development and trial of vaccines against *Brucella*. *J. Vet. Sci.* 18: 281-290.

Mirkalantari S, Zarnani AH, Nazari M, Irajian GR, et al. (2017). *Brucella melitensis*VirB12 recombinant protein is a potential marker for serodiagnosis of human brucellosis. *Ann. Clin. Microbiol. Antimicrob*. 16: 8.

Nagata T, Aoshi T, Uchijima M, Suzuki M, et al. (2004). Cytotoxic T-Lymphocyte-, and Helper T-Lymphocyte-Oriented DNA Vaccination. *DNA Cell Biol.* 23: 93–106.

Neta AVC, Mol JPS, Xavier MN, Paixão TA, et al. (2010). Pathogenesis of bovine brucellosis. *Vet. J.* 184: 146–155.

Olsen SC and Stoffregen WS (2005). Essential role of vaccines in brucellosis control and eradication programs for livestock. *Expert Rev. Vaccines.* 4: 915–928.

Oñate AA, Céspedes S, Cabrera A, Rivers R, et al. (2003). A DNA vaccine encoding Cu, Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. *Infect. Immun.* 71: 4857–4861.

Pollak CN, Wanke MM, Estein SM, Delpino MV, et al. (2015). Immunization with *Brucella* VIRB Proteins Reduces Organ Colonization in Mice through a Th1-Type Immune Response and Elicits a Similar Immune Response in Dogs. *Clin. Vaccine Immunol.* 22: 274–281.

Rivers R, Andrews E, González-Smith A, Donoso G, et al. (2006). *Brucella abortus*: inmunidad, vacunas y estrategias de prevención basadas en ácidos nucleicos. *Arch. Med. Vet.* 38: 7–18.

Rolán HG, den Hartigh AB, Kahl-McDonagh M, Ficht T, et al. (2008). VirB12 is a serological marker of *Brucella* infection in experimental and natural hosts. *Clin. Vaccine Immunol.* 15: 208–214.

Rolán HG and Tsolis RM (2008). Inactivation of the type IV secretion system reduces the Th1 polarization of the immune response to *Brucella abortus* infection. *Infect. Immun.* 76: 3207–3213.

Seleem MN, Boyle SM and Sriranganathan N (2010). Brucellosis: A re-emerging zoonosis. *Vet. Microbiol.* 140: 392–398.

Sieira R, Comerci DJ, Pietrasanta LI and Ugalde RA (2004). Integration host factor is involved in transcriptional regulation of the *Brucella abortus*VIRB operon. *Mol. Microbiol.* 54: 808–822.

Sislema-Egas F, Céspedes S, Fernández P, Retamal-Díaz A, et al. (2012). Evaluation of protective effect of DNA vaccines encoding the BAB1_0263 and BAB1_0278 open reading frames of *Brucella abortus* in BALB/c mice. *Vaccine.* 30: 7286–7291.

Snapper CM, Finkelman FD and Paul WE (1988). Regulation of IgG1 and IgE Production by Interleukin 4. *Immunol. Rev.* 102: 51–75.

Yousefi S, Tahmoorespur M and Sekhavati MH (2016). Cloning, expression and molecular analysis of Iranian *Brucella melitensis* Omp25 gene for designing a subunit vaccine. *Res. Pharm. Sci.* 11: 412-418.

Zhao L, Mahony D, Cavallaro AS, Zhang B, et al. (2016). Immunogenicity of outer membrane proteins VirB9-1 and VirB9-2, a novel nanovaccine against *Anaplasma marginale*. *PLoS One*. 11(4): e0154295.

